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Interactions between E6AP and E6 proteins from alpha and beta HPV types

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ABSTRACT

High-risk mucosotropic Human papillomaviruses (HPVs), especially HPV-16, are the aetiological agents of cervical cancer and the cellular targets of their E6 oncoproteins have been much studied. However, much less is known about the cellular targets of the cutaneous HPV E6 proteins. In this study, a proteomic analysis of cells transfected with the E6 proteins from cutaneous HPV types specifically identified E6-interacting proteins involved in the ubiquitination pathways. These include the E3 ubiquitin-protein ligases E6AP and UBR4/p600. We also show that E6AP can contribute towards the steady-state levels of E6 and, conversely, that certain E6 proteins, in addition to those derived from the high-risk mucosal HPV types, can enhance levels of E6AP turnover. These results define important differences and commonalities in how HPV E6 proteins of mucosal and cutaneous origin interact with cellular ubiquitin-protein ligases.

Introduction

Human papillomaviruses (HPVs) are small DNA viruses that infect epithelial tissue and whose life cycles depend upon the differentiation programme of the epithelium. More than 100 different HPV types have been described and they have very diverse anatomical sites of infection. HPVs are found in the alpha, beta and gamma branches of the papillomavirus phylogenetic tree (de Villiers et al., 2004; Bouvard et al., 2009) The mucosotropic HPV types are found in the alpha group and are generally referred to as high-risk or low-risk, depending upon their association with anogenital malignancies, including cervical cancer. The high-risk HPV-16, for example, is the causative agent of approximately 70% of all cases of cervical cancer. E6 oncoproteins from the high-risk mucosotropic HPV types can induce the degradation of certain cellular target proteins, such as the p53 tumour suppressor and the apoptosis-inducing Bak protein, through interaction with the E6AP ubiquitin-protein ligase (Huibregtse et al., 1993; Scheffner et al., 1993; Thomas and Banks, 1998; Liu and Baleja, 2008). They also induce the degradation of PDZ-containing substrates, possibly in an E6AP-independent manner (for review, see Thomas et al., 2008) E6 proteins from certain of the low-risk mucosotropic HPV types had also been shown to interact with E6AP, albeit with much lower affinity (Storey et al., 1998; Brimer et al., 2007), although their target proteins, apart from Bak (Thomas and Banks, 1999), are as yet unknown. In addition, Pim et al. (2002) had shown that E6s from low-risk mucosotropic HPV types could induce the degradation of PDZ-containing proteins, if they were provided with a PDZ-binding domain, whereas cutaneous E6 proteins could not. Certain cutaneous HPV E6 proteins also interact with Bak (Jackson et al., 2000; Leverrier et al.,2007; Underbrink et al., 2008) and with p300 (Müller-Schiffmann et al., 2006; Muench et al., 2010) whose degradation they enhance (Howie et al., 2011). To further investigate the cellular targets of the cutaneous HPV E6 proteins we initiated a proteomic analysis.

Results

Proteomic analysis of cutaneous HPV E6 proteins

In comparison with the mucosotropic HPV E6 proteins, less is known about the cellular partners of the E6 proteins from cutaneous HPV types. Therefore we performed a proteomic analysis of their potential cellular binding partners. To do this, we transfected 293 cells with plasmids expressing HA-tagged E6 proteins from the cutaneous beta types HPV-8, HPV-24 and HPV-38, and cutaneous alpha type HPV-10. High-risk mucosotropic alpha type HPV-16 E6 and empty vector were included as controls. After 24 h the soluble proteins were extracted from the cells with lysis buffer, and the lysates were incubated on a rotating wheel for 3 h at 4 °C with

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Table 1
Highest-ranking cellular protein specifically pulled down by each HPV E6 protein. By comparison with pulldown from cells transfected with empty vector.

HPV E6	Protein pulled down	Log (*e*)	No. of peptides
HPV-8	p300	−245.1	22/26
HPV-10	E6-AP	−125.1	12/12
HPV-24	E6-AP	−47.0	6/6
HPV-38	UBR4/p600	−90.5	11/11
HPV-16	E6-AP	−41.8	4/4

Log *(e)*: the base-10 log of the expectation that any particular protein assignment was made at random (*E*-value). No. of peptides: the number of different peptides found for this protein/the total number found for this protein.

anti-HA antibody immobilised on agarose beads (EZEView, Sigma). After extensive washing and removal of detergent, the beads were subjected to tryptic digest and mass spectroscopy.

The pulldown profile of each E6 protein was compared with that of the empty vector pulldown, to eliminate non-specific protein interactions, and the highest-ranked, specifically pulled-down protein for each E6 is shown in Table 1. In each case, the relevant E6 protein was detected, albeit at low levels. A positive control for the assay was provided by the HPV-8 E6, whose highest affinity pulldown partner was p300, which has been previously published to be a specific interacting partner of HPV-8 E6 (Müller-Schiffmann et al., 2006; Muench et al., 2010; Howie et al., 2011). The highest affinity pulldown partner of HPV-38 E6 was the UBR4 ubiquitin ligase, also known as p600, which has been shown to be targeted by the E7 proteins of HPV-16, HPV-6b, HPV-11 and bovine papillomavirus (BPV-1) (Huh et al., 2005; DeMasi et al., 2005). Another ubiquitin ligase, E6AP was the highest affinity pulldown partner of both HPV-10 and HPV-24 E6. E6AP was first described as an important cellular partner of the high-risk mucosotropic HPV E6 proteins, and it was initially thought to be an interaction specific to cancer-related HPV types (Huibregtse et al., 1993; Scheffner et al., 1993). However, the low risk HPV 11E6 has since been shown to also interact with E6AP (Storey et al., 1998; Brimer et al., 2007). Since the interactions between high-risk mucosal HPV E6 proteins and E6AP had been shown to affect the levels of each protein in vivo (Kao et al., 2000; Tomaić et al., 2009a), it was clearly of interest to determine the possible functions of this interaction in the case of cutaneous HPV E6 proteins.

E6AP protein levels in the presence of E6 in vivo

It has long been known that HPV-16 E6 can induce the proteasomal degradation of E6AP (Kao et al., 2000), so we first investigated the stability of E6AP in the presence of E6 proteins from a range of HPV types. HEK293 cells were transfected with a plasmid expressing the E6AP wild type or the $c > a$ mutant, which is catalytically inactive (Huibregtse et al., 1995), plus plasmids expressing a series of HPV E6 proteins. After 24 h the cells were harvested and the cell extracts analysed by SDS-PAGE and Western Blot. The blots were probed with anti-E6AP antibody and the results are shown in Fig. 1, and as can be seen the levels of E6AP are reduced in the presence of the alpha type HPV E6 proteins 10, 11, 16; but not in the presence of the beta type HPV E6 proteins 8, 12, 14, 24, 38. Interestingly, the $c > a$ mutant responds very similarly to the wild type, which is consistent with previous reports implicating recruitment of endogenous wild type E6AP to direct the degradation of this mutant (Kao et al., 2000).

It was possible that the reduction in E6AP levels seen with the alpha type HPV E6 proteins was caused by proteasomal degradation of the E6AP protein, or alternatively that the E6AP was

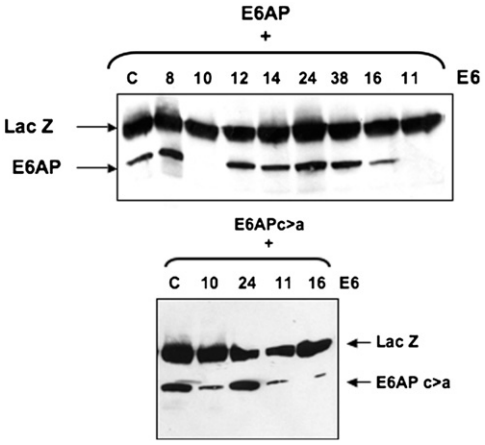

Fig. 1. E6AP levels in the presence of E6. Western blots of HEK293 cells transfected with E6AP wild type (upper panel) or c > a mutant (lower panel), either alone (C) or with the E6 proteins from HPV 8, 12, 14 and 24 (beta types) and HPV 10, 11 and 16 (alpha types). Blots were probed with anti-E6AP antibody plus anti-β-galactosidase (LacZ) as control.

relocated into an insoluble fraction of the cell. To address these possibilities, we repeated the transfection and western blot with E6AP and the HPV-10, HPV-11 or HPV-16 E6 proteins, including proteasome inhibitors. We also analysed E6AP levels in both the E1a extract (“soluble fraction”) and in the residue (“insoluble fraction”).

The results are shown in Fig. 2, where it can be seen that the levels of E6AP in the insoluble fraction are extremely low and do not change in the presence of HPV-10, HPV-11 or HPV-16 E6, with or without proteasome inhibition. Thus, the changes seen in E6AP levels do not appear to be caused by relocation of the protein.

It can also be seen in the soluble fraction that proteasome inhibition can rescue the reduction in E6AP levels caused by HPV-16 E6, as might be expected from previous reports (Kao et al., 2000). Interestingly, the HPV-11 E6-induced reduction of E6AP is also rescued to a certain extent, but the HPV-10 E6 effect upon E6AP levels appears to be, at least in part, proteasome-independent.

Half life of E6AP in the presence of E6

Clearly, the presence of HPV-10 and HPV-11 E6 resulted in lower levels of E6AP in the cell, and we wished to determine whether this was the result of increased turnover of E6AP protein, as is the case with the high-risk HPV-16 and HPV-18 E6s. To address this, we performed a half-life assay, using E6AP-null cells to reduce any potential interference with endogenous E6AP (Massimi et al., 2008; Tomaić et al., 2009a). Briefly, cells were transfected with E6AP either alone or together with HPV-10 E6 or HPV-11 E6. After 24 h the cells were treated with cycloheximide at 50 μg/ml in DMSO to block further protein synthesis. The cells were harvested at 0, 2, 4, 7, 14 and 21 h post-treatment and the levels of E6AP analysed by SDS-PAGE and western blot. The results are shown in Fig. 3, where it can be seen that the half-life of E6AP in the absence of E6 is between 14 and 21 h, as reported previously (Tomaić et al., 2011). It is also clear that the half-life of E6AP is reduced to approximately 2 h in the presence of HPV-10 E6 and around 7 h with HPV-11 E6. Thus, the reduction in E6AP levels seen in the presence of HPV-10 and HPV-11 E6 proteins (Figs. 1 and 2) would appear to be the result of destabilisation of the E6AP protein.

E6AP stability in the presence of E6 in vitro

Having shown that the E6 proteins from HPV-10, HPV-11 and HPV-16 could induce the degradation of exogenous E6AP in

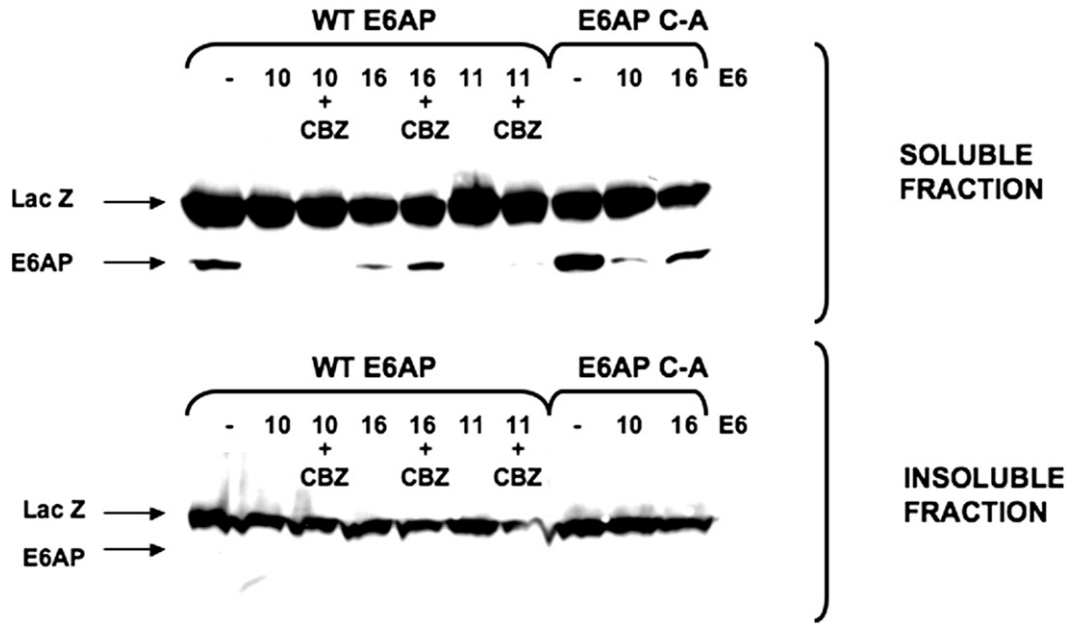

Fig. 2. Effect of proteasome inhibition upon E6-induced reduction of E6AP levels. Western blots of HEK293 cells transfected with E6AP wild type or $c > a$ mutant, together with the indicated E6 proteins. Prior to harvesting the transfected cells were treated with proteasome inhibitors CBZ and LLnL (CBZ) for 2 h. Blots were probed with anti-E6AP antibody plus anti-β-galactosidase (LacZ) as control.

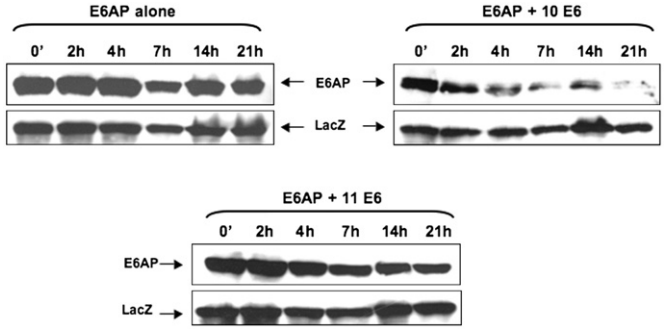

Fig. 3. E6AP half-life is reduced in the presence of HPV-10 and HPV-11 E6 proteins. E6AP-null cells were transfected with plasmids expressing E6AP and either HPV-10 E6 or HPV-11 E6. After 24 h the cells were treated with cycloheximide, harvested at the times indicated, and analysed by SDS-PAGE and Western blot. Blots were probed with anti-E6AP antibody plus anti-β-galactosidase (LacZ) as control.

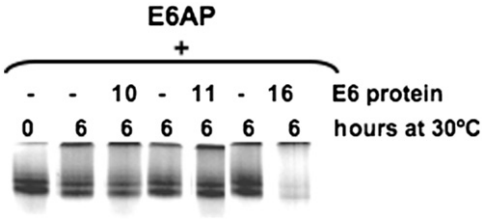

Fig. 4. E6AP stability in the presence of E6 in vitro. In vitro translated radiolabelled E6AP protein was mixed with water-primed lysate (−), or with in vitro translated radiolabelled E6 protein as indicated and incubated for 6 h at 30 °C, then anaysed by SDS-PAGE and autoradiography.

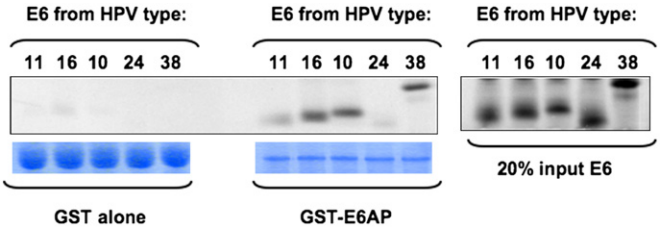

Fig. 5. Binding of E6 proteins to E6AP in vitro. In vitro translated, radiolabelled E6 protein, as indicated, was mixed with GST alone or with GST-E6AP fusion protein immobilised on glutathione agarose (Sigma) and incubated at 4 °C for 2 h, then washed extensively and analysed by SDS-PAGE and autoradiography. The upper panels show the autoradiographs; the lower panels show the Coomassie-stained gel.

transfected cells, it was of interest to know whether they could induce its degradation in vitro. To address this, we performed an in vitro degradation assay. In vitro translated radiolabelled E6AP protein was mixed with water primed lysate, or with in vitro translated HPV-10, HPV-11 or HPV-16 E6 and incubated at 30 °C for 6 h, then analysed by SDS-PAGE and autoradiography. The results in Fig. 4 clearly show that HPV-16 E6 induces significant degradation of E6AP in vitro; no degradation is seen with HPV-11 E6 and a very slight reduction of E6AP levels is seen with HPV-10 E6.

Binding of E6 proteins to E6AP in vitro

It was clear from the assays both in vitro and in vivo that the E6 proteins do not interact identically with E6AP. To examine the binding activities in more detail, we performed in vitro binding assays with either GST alone, or GST-E6AP and in vitro translated HPV E6 proteins. The results of these assays are shown in Fig. 5, where it can be seen that HPV-16 E6 binds strongly to GST-E6AP and HPV-11 E6 binds weakly in vitro, as would be expected from previous studies (Storey et al., 1998; Brimer et al., 2007). The HPV-10 and HPV-38 E6 proteins bind strongly to the GST-E6AP, whereas the HPV-24 E6 scarcely binds E6AP at all in vitro. This is in interesting contrast to the results from the pulldown assay in which HPV-24 E6 bound strongly to E6AP, and HPV-38 E6 did not bind E6AP, the latter of which is in agreement with previous studies (White et al., 2012). This suggests that there may be proteins, or protein modifications, involved in the in vivo binding

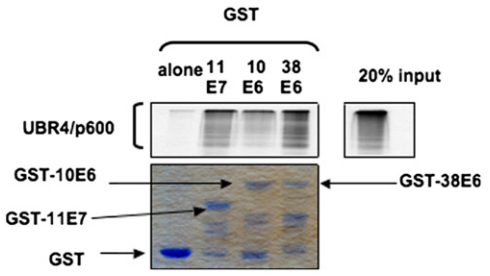

Fig. 6. HPV-38E6 binds UBR4/p600 in vitro. In vitro translated, radiolabelled UBR4/p600 protein was mixed with GST alone, GST-11E7, GST-10E6 or GST-38E6 fusion protein immobilised on glutathione agarose (Sigma) and incubated at 4 °C for 2 h, then washed extensively and analysed by SDS-PAGE and autoradiography. The upper panels show the autoradiographs; the lower panels show the Coomassie-stained gel.

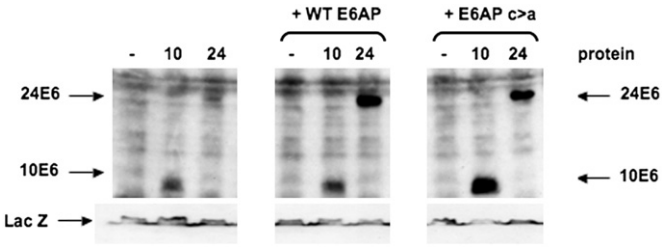

Fig. 7. E6AP stabilises E6 *in vivo*. Western blot of HEK293 cells transfected with the indicated HA-tagged E6s, either alone, with wild-type E6AP or with the $c > a$ mutant of E6AP. The blot was probed with anti-HA antibody, plus anti − β-galactosidase (LacZ) as control. The HA-tagged E6 proteins are arrowed.

of HPV-24 E6 to E6AP that are not present in the in vitro translated protein preparation.

A strong interacting partner of HPV-38 E6 in vivo appeared to be UBR4/p600 (Table 1). UBR4/p600 had been shown to interact with the E7 proteins of HPV-16, HPV-6b, HPV-11 and bovine papillomavirus (BPV-1) (Huh et al., 2005; DeMasi et al., 2005), but no interaction with an HPV E6 protein had been confirmed. To investigate this we performed an in vitro binding assay, using in vitro translated, radiolabelled UBR4/p600 and GST alone, GST-11E7 as positive control, GST-10E6, and GST-38E6. The results are shown in Fig. 6, where it can be seen that UBR4/p600 binds to GST-38E6 at a comparable level to its binding of GST-11E7, thus confirming the results of the proteomic analysis.

E6AP stabilises E6 proteins in vivo

Previous studies have shown that one consequence of the interaction between HPV E6 and E6AP is stabilisation of the E6 protein (Tomaić et al., 2009a). To investigate the effects of E6AP upon the expression levels of HPV-10 and HPV-24 E6, we transfected 293 cells with vectors expressing HA-tagged HPV-10 and HPV-24 E6 proteins, with or without a vector expressing either wild type E6AP, or the $c > a$ mutant of E6AP. After 24 h we analysed the E6 proteins by SDS-PAGE and western blotting and the results are shown in Fig. 7. It can clearly be seen that the levels of HPV-24 E6 markedly increase in the presence of both wild-type and mutant E6AP, while HPV-10 E6 levels are somewhat increased with wild-type E6AP, and strongly increased in the presence of the catalytically-defective mutant of E6AP. It had previously been shown that the HPV-16 and HPV-18 E6s were stabilised in the presence of E6AP (Tomaić et al., 2009a), and our finding that taxonomically diverse cutaneous HPV E6 proteins are also stabilised suggests that interaction with E6AP is a means of stabilising the E6 protein that is common to many HPV types.

Discussion

A protein pulldown assay with the E6 proteins from a number of evolutionarily diverse HPV types was used as a starting point to investigate their potential cellular protein-binding partners. The pulldown was validated by the strong pulldown of p300 by HPV-8 E6 (Müller-Schiffmann et al., 2006; Muench et al., 2010) and E6AP with HPV-16 E6. The pulldown of E6AP with both HPV-10 and HPV-24 E6 proteins was somewhat surprising, in that no ubiquitin/proteasomal activities had previously been associated with the E6 proteins of these virus types, but the co-pulldown of several proteasome subunits (data not shown) in each case further validated the result. The pulldown of UBR4/p600 with HPV-38 E6 was also surprising, since previous reports had shown UBR4/p600 to be targeted by E7 proteins rather than E6. (Huh et al., 2005; DeMasi et al., 2005). In fact, a recent proteomic analysis also suggests that HPV-38 E6 might bind UBR4/p600, but that observation was not validated (White et al., 2012), Here we have shown that the GST HPV-38 E6 can bind to in vitro translated UBR4/p600 as strongly as its known interactor, HPV-11 E7. No significant binding is seen with HPV-10 E6, indicating that this interaction may be specific to HPV-38 E6. Possibly certain beta-type HPVs, such as HPV-38, target UBR4/p600 mainly through their E6 protein, similar to the case of Rhesus papillomavirus which targets polarity proteins through a PDZ-binding motif on the E7 protein, rather than on the E6, as in high-risk HPV types (Tomaić et al., 2009b).

We have shown here that E6AP can interact with diverse alpha and beta type HPV E6 proteins, regardless of their cancer association, and that this interaction can increase the steady-state levels of those E6 proteins. However there are clear differences in the effects of the interaction upon the E6AP protein. Half-life analyses show that the E6 proteins of alpha HPV-10, HPV-11 (Fig. 3) and HPV-16 (Kao et al., 2000) can all reduce the stability of E6AP in vivo, whereas the ability to induce E6AP degradation in vitro appears to be restricted to the high-risk alpha HPV-16 E6 with only weak activity seen with HPV-10 E6. In addition, only the HPV-16 E6-induced degradation of E6AP is significantly blocked by proteasome inhibition, which is consistent with the findings of Kehmeier et al. (2002). Thus, it would seem that the low risk alpha E6 proteins can induce the degradation of a target protein, but in a manner different from the high-risk alpha E6s. This supports the conclusions of Pim et al. (2002), who found that low-risk HPV E6 proteins could induce the degradation of PDZ-containing proteins when supplied with a PDZ-binding motif in the form of a chimæric tail.

From the proteomic analysis it was clear that the various E6s interacted to different degrees with E6AP; in GST pulldown assays with in vitro translated E6 proteins these differences were more marked. HPV-11 E6 bound GST-E6AP more weakly than HPV-16 E6, as might have been expected from previously published data (Storey et al., 1998; Kao et al., 2000; Brimer et al., 2007). However HPV-10 E6 and HPV-38 E6 proteins both bound as strongly as the HPV-16, while HPV-24 E6 barely bound GST-E6AP in vitro. This was in contrast to the results from the mass spectroscopy, in which HPV-24 E6 bound strongly to E6AP, while HPV-38 E6 did not. This may suggest that the HPV-24 E6 is subject to post-translational modifications that enhance E6AP interaction, but which do not occur in the reticulocyte lysate translation system. This is supported by the fact that, as can be seen from Figs. 5 and 7, the in vitro translated HPV-24 E6 runs with slightly greater mobility than the other E6 proteins, whereas in vivo it runs much more slowly. The discrepancy between the in vitro and in vivo results with HPV-38 E6 is harder to explain, but one intriguing possibility is competition between UBR4/p600 and E6AP for the same site of interaction on HPV-38 E6. Since

UBR4/p600 appears to be a very strong interactor in the proteomic analysis, this may explain why E6AP is only bound in the in vitro assay.

Finally, we show that in the presence of E6AP the stability of the HPV-10 and HPV-24 E6 proteins is enhanced. This is consistent with the findings of Tomaić et al. (2009a) who had shown similar enhanced stability of the high risk mucosotropic HPV-16 and HPV-18 E6 proteins. However, regardless of the strength with which the different E6 proteins appear to target E6AP for degradation, the effects on E6 stability appear similar. This raises the issue of how a protein that is being degraded by E6 can nonetheless enhance E6 stability. However, many protein-protein interactions can result in active protein stabilisation. This can be through chaperone-like activity (DeFee et al., 2011) or in the case of proteasome-linked activity, by potential protection at the site of the proteasome itself (Coleman et al., 2003). Current studies aim to elucidate which of these possiblities is the most likely for the E6-E6AP complex.

Materials and methods

Cells

The cells used were HEK293 cells, maintained in Dulbecco's modified medium supplemented with 10% foetal calf serum.

Plasmids and transfections

The plasmids expressing HA-tagged E6 proteins and the untagged E6 proteins for in vitro expression have been described previously (Massimi et al., 2008), as have the plasmids expressing wild type and mutant E6AP and GST-E6AP (Tomaić et al., 2009a). The plasmid expressing V5-tagged UBR4/p600 was the kind gift of Dr Takafumi Tasaki.

Transfections were done using the standard calcium phosphate precipitation method (Wigler et al., 1979) and the cellular proteins were extracted in E1a buffer after an overnight incubation, as described previously (Thomas et al., 2002)

Western blots and antibodies

Western blots were performed on cell extracts as described previously (Massimi et al., 2008). The primary antibodies used were anti-HA (Roche) at 1/1000 and anti-β-galactosidase (Promega) at 1/5000 and anti-E6AP (BD Biosciences) at 1/1000; HRP-conjugated secondary antibodies (DAKO) were used as appropriate.

In vitro translation and degradation assays

Proteins were translated in vitro using the Promega TnT in vitro translation kit according to the manufacturer's instructions, and radiolabelled with [35*S*]-cysteine (Perkin Elmer). The in vitro degradation assays were performed as described previously (Thomas et al., 2002), but using a 6 h incubation.

GST pulldown assays

These were performed as described previously (Massimi et al., 2008).

Half-life assays

These were performed as described previously (Tomaić et al., 2011).

Pulldown and mass spectroscopy

HEK293 cells were transfected with plasmids expressing HA-tagged E6 proteins (Massimi et al., 2008), or with empty vector, by calcium precipitation. After overnight incubation the cells were extracted in mass spectroscopy lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 0.25% NP40) containing protease inhibitor cocktail I (Calbiochem) and 0.15 mg/ml dextran, as described before (Tomaić et al., 2009b). The extracts were then incubated with anti-HA-agarose beads (Sigma) for 3 h at 4 °C on a rotating wheel. The beads were extensively washed, dried and subjected to mass spectroscopy.

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